

REMARKS

Applicant notes with appreciation the withdrawal of the objection to claim 17 as articulated on page 4, section 5 of the Final Office Action (Paper No. 20051007) as well as the rejection of claims 17-28 under 35 U.S.C. §112, second paragraph, as articulated on page 8, section 7 of the Final Office Action (Paper No. 20051007).

Currently, claims 9-28 remain pending in the application.

Currently, claims 9 and 10 stand rejected under 35 U.S.C. §102(b) over Loyola-Rodriguez et al. (J. Gen. Microbiol., 138:269-274, 1992), Ikeda et al., (Infection and Immunity, 35:861-868, 1982) and Ooshima et al., (Microbiol. Immunol., 29:1163-1173, 1985). Claims 9-28 stand rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement.

Remarks Directed to Rejection of Claims 9 and 10 under 35 U.S.C. §102(b) as Anticipated by Loyola-Rodriguez et al., Ikeda et al. and Ooshima et al.

The basis of the rejection relating to Loyola-Rodriguez et al. was originally set forth in Paper No. 20050423 and Paper No. 20051007 and is reiterated in the outstanding Office Action (Paper No. 20060801, page 3 – page 5, section 4). The rejection relating to Ikeda et al. and Ooshima et al. is set forth in the outstanding Office Action on pages 15-16, section 7.

The Examiner states that “Loyola-Rodriguez et al. teach a method of treating rats against infection caused by Streptococcus mutans by administering mutacin” and that “[t]he amino acid sequence as set forth in SEQ ID No:2 would be inherent in the teachings of the prior art.” (Paper No. 20060801, page 3, section 4) In spite of the clear difference between the molecular weight of the protein described by Loyola-Rodriguez et al., 6500 Daltons, and that of the present invention, the Examiner directs attention to the word “comprising” used in these claims as a basis for the position that “the prior art teaches the claimed method.” Although the relationship between the “comprising” language and the asserted inherency of the claimed method in the prior art is unclear, Applicant assumes for purposes of this response that the Examiner believes that the clearly larger protein described by Loyola-Rodriguez et al. is a larger form of a mutacin which includes the protein of SEQ

ID NO. 2.

As noted previously, the law as to inherent anticipation is well established in requiring that the missing element absolutely must be present in the thing described in the reference and not merely probable or possibly present. In *Rosco Inc. v. Mirror Lite Co.*, 64 USPQ2d 1676, 1680, the court has stated:

Under the doctrine of inherency, if an element is not expressly disclosed in a prior art reference, the reference will still be deemed to anticipate a subsequent claim if the missing element "is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill." *Cont'l Can Co. v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991). "Inherent anticipation requires that the missing descriptive material is 'necessarily present,' not merely probably or possibly present, in the prior art." *Trintec Indus., Inc. v. Top-U.S.A. Corp.*, 295 F.3d 1292, 1295, 63 USPQ2d 1597, 1599 (Fed. Cir. 2002) (quoting *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999)).

Applicant believes that given the teachings of this reference, there is no basis for the assertion that the protein of SEQ ID NO. 2 is necessarily present in the protein described by Loyola-Rodriguez et al.

In particular, although the Examiner states that "[t]he peptide used in the claimed method is isolated and purified from *Streptococcus mutans*..." and "[t]hese properties are taught by the peptide of the prior art." (Paper No. 20060801, section 4, p.4), Applicant notes that the polypeptide described in Loyola-Rodriguez et al. is isolated from *S. sobrinus*, not *S. mutans*. (Loyola-Rodriguez et al., Abstract)

The distinction between the source of MT6223 and currently claimed polypeptides has been previously pointed out to the Examiner in section 8 of the Declaration by Dr. Page Caufield which definitively states that MT6223, the polypeptide described in Loyola-Rodriguez et al., "is isolated from *Streptococcus sobrinus* while mutacin I, is isolated from *Streptococcus mutans*." A copy of the Declaration is again submitted herewith for the Examiner's reference.

Thus, it is submitted that the protein disclosed in Loyola-Rodriguez, MT6223, is not identical to the protein of SEQ ID NO. 2 based on the identification of these proteins as having different molecular weights, among other physical distinctions. Further, these two proteins are isolated from

different species, not from the same source as asserted by the Examiner. Thus, it is submitted that there is no reasonable basis for the assertion that the protein of SEQ ID NO:2 is necessarily present in MT6223 as is required to reject a claim as anticipated based on inherency.

Turning to the disclosures of cited references Ikeda et al.; and Ooshima et al., Applicant notes that these references both describe a protein isolated from *S. mutans* C3603. The Ikeda et al. article describes the protein as "bacteriocin C3603." (Abstract and throughout) The Ooshima et al. article refers to the Ikeda et al. article and states that "[b]acteriocin C3603 was isolated from the culture supernatant of *S. mutans* C3603 (serotype c) as described previously (7)" where (7) indicates reference to the Ikeda et al. article cited by the Examiner. (Ooshima et al., "Materials and Methods," first sentence and "References") Thus, it is submitted that the two articles, Ikeda et al.; and Ooshima et al., refer to the same protein "bacteriocin C3603."

Applicant submits that the protein bacteriocin C3603 is not equivalent to the protein of SEQ ID NO:2 described in the present specification and included in claims 9 and 10. For example, the molecular weight of bacteriocin C3603 is cited as 4800 Daltons (Ikeda et al., Abstract) which contrasts with the smaller size of the protein of SEQ ID NO:2 described in the present specification.

In addition, careful reading of these references shows that it is logically inconsistent to maintain that the protein bacteriocin C3603 could inherently "include" the protein of SEQ ID NO:2. Ikeda et al. includes the information that "...bacteriocin C3603 contains aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, tyrosine, phenylalanine, tryptophan, lysine and arginine." (page 866, col. 1) In contrast, the present specification shows that the protein of SEQ ID NO:2 contains leucine, cysteine, asparagine and proline. Applicant submits that the protein of Ikeda et al. and Ooshima et al. could not "contain" the protein of SEQ ID NO:2 without also containing the amino acids leucine, cysteine, asparagine and proline.

In summary, in view of the clearly defined physical differences between the proteins described in the cited references and that of SEQ ID NO:2, as well as the differing sources of those proteins, Applicant submits that the Examiner has not established that any of the references describes a protein that necessarily includes the protein of SEQ ID NO:2 and therefore the references do not inherently disclose the protein of SEQ ID NO:2. Since it is well established that "[t]o anticipate a claim, the reference must teach every element of the claim," (MPEP 2131), Applicant submits that the references are not anticipatory.

In light of the above remarks, reconsideration and withdrawal of the rejection as to claims 9 and 10 under 35 U.S.C. §102(b) over Loyola-Rodriguez et al., Ikeda et al. and Ooshima et al. is respectfully requested.

**Remarks Directed to Rejection of
Claims 9-22 under 35 U.S.C. §112, First Paragraph**

A first basis of the rejection indicating failure to comply with the enablement requirement is that the claims are drawn to a method of treating or preventing all Gram-positive bacterial infections.

Applicant notes that claims are enabled when one of skill in the art would know how to make and use the claimed invention without undue experimentation given the disclosures in the patent application coupled with information known in the art. MPEP2164.01 It is submitted that the present claims are believed to meet this standard and are therefore enabled.

Applicant further notes the Examiner's burden in rejecting claims as unenabled under 35 U.S.C. §112, first paragraph, detailed in the Manual of Patent Examining Procedure section 2164.04, includes, at a minimum, giving reasons for the uncertainty of the enablement. Specifically, it is the Examiner's burden to provide "factors, reasons and evidence that lead the examiner to conclude that the specification fails to teach how to make and use the claimed invention without undue experimentation..." (MPEP 2164.04) Still more specifically, this section suggests "specific findings of fact, supported by evidence, and then drawing conclusions based on these findings of fact."

Applicant submits that the Examiner has not formed a proper rejection under 35 U.S.C. §112, first paragraph as required. Applicant submits that instead of providing "factors, reasons and evidence that lead the examiner to conclude that the specification fails to teach how to make and use the claimed invention without undue experimentation..." the Examiner has simply cited references which describe various pathogenic bacteria and noted that the present specification does not specifically include information regarding these bacteria. The Examiner has not provided information showing that one of skill in the art would not know how to make and use the claimed invention with reference to any particular Gram positive organism.

Applicant submits that information disclosed in the present application enables the claims. In particular, Applicant notes the statements in the specification supporting the current claims to "a method of treating or preventing a gram-positive infection" (claim 9) and "a method of treating or

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preventing an infection in a subject, said method comprising administering to said subject infected with or susceptible to a gram-positive bacterium selected from the genus consisting of Staphylococcus, Enterococcus, and Streptococcus pneumoniae.” (claim 17) For example, the specification states that mutacin I has advantages compared to conventional antimicrobial agents including that “it has a wide spectrum of antimicrobial activity against a wide range of gram-positive bacteria including the multidrug resistant Staphylococci and Enterococci...” (page 18, lines 1-4). The specification also states that “mutacin III is more potent against Staphylococcus aureus and Staphylococcus epidermidis, while both mutacins have equal activities against other pathogens such as enterococci, pneumococci, and Group A streptococci.” (page 33, lines 10-13)

Applicant submits herewith a Declaration by an inventor, Dr. Page Caufield including data showing particular Gram positive organisms to the action of mutacin I and supporting the disclosure of a wide spectrum of antimicrobial activity against a wide range of gram-positive bacteria including the multidrug resistant Staphylococci and Enterococci...” The Declaration includes data and description of effectiveness of mutacin I inhibition against various Gram positive organisms along with information indicating that “[b]ased on the broad spectrum of mutacin I to inhibit pathogenic gram-positive bacteria, I believe that one [of my colleagues] of skill in the field would have little difficulty in using or testing the efficacy of mutacin I against a Gram positive bacterial target...” (Declaration, section 5) Applicant submits that the Declaration supports the assertion that undue experimentation is not required to make and use the methods of the present claims.

In addition, Applicant submits that testing the susceptibility of a particular microorganism to an inventive peptide is well within the talents of one of skill in the art as evidenced by published results. In support of this position, Applicant refers to the Loyola-Rodriguez reference for an exemplary teaching with respect to Table 2 of methodologies for measuring the level of success.

Further, testing the activity of even well-known antibiotic compositions on a lab sample from a patient is submitted to be a routine part of medical practice. Applicant submits an article supporting this assertion entitled “Agar Plate Dilution Method for Routine Antibiotic Susceptibility Testing in a Hospital Laboratory” (A. J. Clinical Pathol., 60:384-394, 1973) (see Appendix B).

Thus, Applicant believes that the disclosure provides adequate guidance and that any experimentation necessary would be routine and not undue experimentation. It is therefore submitted

that the current claims are enabled since one of ordinary skill in the art would be able to make and use the invention without undue experimentation.

A second basis of the rejection indicating failure to comply with the enablement requirement is that the claims refer to "prodrugs" which the Examiner interprets as meaning a "variant" of SEQ ID NO:2. (Paper No. 20060801, pages 11-12, section 6)

Applicant submits that there is no basis for the rejection of the claims as unenabled as to prodrugs as recited in the claims. The Examiner's cited references, Creighton, 1984, Creighton, 1989 and Nosoh, 1991, do not appear to be relevant in the context of prodrugs as understood in the art or as defined in the specification since the references appear to be directed to changes in primary amino acid structure such as amino acid substitution achieved through bioengineering.

The instant specification defines the term "prodrug" as referring "to compounds that are rapidly transformed in vivo to yield the parent compounds of the above formula, for example, by hydrolysis in the blood." (p.21, lines 5-7, emphasis added) The specification provides further information pertaining to prodrugs, incorporating by reference "T. Higuchi and V. Stella, "Prodrugs as Novel Delivery systems," Vol.14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987." (p.21, lines 7-10) It is submitted that one of skill in the art would not recognize an amino acid substitution as a prodrug since it is not clear how such a protein would be rapidly transformed in vivo to yield the parent compound, that is, the protein of SEQ ID NO:2.

Applicant further directs the Examiner's attention to U.S. Patent No. 6,699,970 which includes a claim directed to "[a]n isolated and purified peptide sequence consisting of: the amino acid sequence as set forth in SEQ ID No: 2, or a pharmaceutically acceptable, ester, amide, and prodrug thereof." Since an issued claim is presumptively valid, and since the same specification supported the quoted claim, Applicant submits that the current claims reciting a "prodrug" are likewise enabled by the current specification.

In support of enablement of pending claims 9-28, Applicants submit the declaration of coinventor Page Caufield inclusive of data indicating the effectiveness of that of SEQ ID No: 2 against a variety of Gram-positive bacteria including Staphylococcus pyogenes, Streptococcus pneumoniae, multiple drug resistant Staphylococcus aureus (MDRSA), vancomycin-resistant

Enterococcus faecium, and Bacillus anthracis. Additionally, Applicant incorporates by reference the remarks made of record on July 28, 2005, pages 7-8.

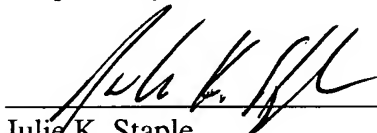
In light of the attached declaration indicating activity of the claimed invention against the broad spectrum of gram-positive bacteria including the most pathogenically aggressive forms, it is respectfully submitted that one of skill in the art would readily be able to test the efficacy and prepare an administrative composition for given gram-positive bacteria based on the teachings found within the specification as filed.

Thus, reconsideration and withdrawal of the rejection as to pending claims 9-28 under 35 U.S.C. §112, first paragraph, for failure to comply with the enablement requirement is requested.

Summary

Claims 9-28 are the claims currently being examined in the application. Of these claims, 9 and 17 are the only independent claims. It is submitted that the claims in their present form are believed to be allowable, and reconsideration and allowance of the claims is solicited.

Respectfully submitted,


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Date: December 4, 2006

Agar Plate Dilution Method for Routine Antibiotic Susceptibility Testing in a Hospital Laboratory

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ABSTRACT

Haltalin, Kenneth C., Markley, Anne H., and Woodman, Edythe: Agar plate dilution method for routine antibiotic susceptibility testing in a hospital laboratory. *Am. J. Clin. Pathol.* 60: 384-394, 1973. The agar plate dilution method for routine antibiotic susceptibility testing has been used in this hospital laboratory since 1968. The experience has met with the approval of the laboratory staff, and the technic has provided clinicians with more precise and useful information than was formerly obtained with disk sensitivity methods. Two studies were performed to document the reliability of our method. Testing of 60 organisms by both agar dilution and broth dilution methods showed that inhibitory concentrations were identical or varied by only one dilution, with a single exception. To compare results obtained using antibiotic laboratory standards and commercial intravenous preparations, 120 strains were tested in duplicate. Minimal inhibitory concentrations with both forms of drugs were similar or varied by only one dilution in all cases. A "time-work" study showed that the agar dilution method can be performed economically in a routine laboratory setting. The agar dilution method is appropriate for use in hospital laboratories and has distinct advantages over the disk agar diffusion technic.

THE VARIOUS TECHNIQUES available for antimicrobial susceptibility testing in diagnostic microbiology laboratories have been critically evaluated in two recent monographs.^{3, 5} Virtually all clinical laboratories use a disk agar diffusion method such as the one described by Bauer and associates.² Determination of antimicrobial susceptibility by dilution technics is assumed by most technologists and laboratory directors to be too complicated, expensive, and time-

consuming for routine use in clinical laboratories. Several years ago, the agar plate dilution method was semiautomated by the introduction of a simple, inocula-replicating apparatus by Steers and associates,⁶ thereby making the technic more feasible for use in diagnostic laboratories. In spite of this, the routine use of the agar dilution method has apparently remained confined to very few clinical laboratories⁶ and to research units.

In mid-1968 an agar plate dilution method utilizing the device of Steers and associates replaced the disk technic as the principal method of performing susceptibility testing in the microbiology laboratory.

Received September 12, 1972; received revised manuscript October 12, 1972; accepted for publication November 15, 1972.

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tory of Children's Medical Center of Dallas.
This communication describes our proce-
dure, evaluates its reliability and efficiency,
and summarizes selected results.

Materials and Methods

Hospital and Microbiology Laboratory

Children's Medical Center is a 122-bed
hospital with full medical and surgical
services and is a major teaching institution
for house staff and medical students of the
Departments of Pediatrics and Surgery of
the University of Texas Southwestern Med-
ical School at Dallas. During 1971 there
were 6,624 hospital admissions (60% pri-
vate and 40% staff) and 63,976 outpatient
visits. There are 176 surgeons and 169 pe-
diatricians on the attending staff.

The microbiology laboratory is staffed by
a supervisor, two medical technologists, one
clerk-laboratory assistant, and a part-time
media-maker. The laboratory is directed by
the chief of pathology; an advisory role is
assumed by a member of the pediatric in-
fectious disease service. Two technologists-
in-training are assigned to the laboratory
at all times and a resident physician or a
medical student is present 1 day a week for
instruction. Approximately 60 specimens
are received each day and about 20 orga-
nisms are tested for antibiotic susceptibil-
ity daily Monday through Friday.

Agar Dilution Method

Gram-negative organisms are tested with
ampicillin (Ayerst Laboratories), cephalo-
thin (Lilly & Company), chloramphenicol
(Parke-Davis & Company), kanamycin (Bris-
tol Laboratories), polymyxin B (Pfizer Lab-
oratories), tetracycline (Lederle Laborato-
ries), and gentamicin (Schering Corpora-
tion) ("Gram Negative Set"). Gram-positive
organisms are tested with penicillin G
(Pfizer Laboratories), methicillin (Bristol
Laboratories), erythromycin (Abbott Lab-
oratories), cephalothin and gentamicin
("Gram Positive Set"). Enterococci, *Listeria*

species, and unusual organisms are tested
with both sets. The commercial prepara-
tions of antibiotics for intravenous adminis-
tration used in the tests are obtained from
the hospital pharmacy at a cost of approxi-
mately \$20.00 per week. Chloramphenicol
is supplied gratis as the free salt by Parke-
Davis. (Chloramphenicol succinate, the
commercial preparation for intravenous ad-
ministration, has no antibacterial action
in vitro).

Once a week fresh antibiotic solutions
are prepared. For chloramphenicol, 100 mg.
of drug are dissolved in 2 ml. of 95% ethyl
alcohol. Other antibiotics are reconstituted
with sterile distilled water. Serial dilutions
are made with distilled water to give the
desired concentrations. One milliliter of a
given concentration of antibiotic is added to
99 ml. of sterile Oxoid Sensitivity Test
Medium* contained in an 8-ounce media
bottle held in a water bath at 45 to 48 C.
Twenty milliliters of agar are poured into
each of five petri plates (100 mm. x 15
mm.). Final antibiotic concentrations in
agar, except for gentamicin and penicillin,
are 20, 10, 5, 1.25, and 0.3 µg. per ml. Agar
concentrations of gentamicin are 10, 5, 2.5,
1.25, and 0.3 µg. per ml., and those of peni-
cillin are 2.5, 1.25, 0.6, 0.2, and 0.08 units
per ml. Plates are left at room temperature
overnight to dry the agar surfaces and then
stored at 4 C. All plates are used within 7
days. Double sets of gentamicin and cepha-
lothin plates are prepared each week, since
these drugs are used in both Gram-nega-
tive and Gram-positive sets. Growth con-
trol plates are poured without antibiotics.

In the morning, seed cultures are pre-
pared by inoculating organisms selected for
testing into screw-top tubes containing 5
ml. of Oxoid Sensitivity Test Broth and in-
cubated at 37 C. for 4 hours. After incuba-
tion 0.01 ml. of broth is removed with a
calibrated loop and placed into 1 ml. of

* Colab Laboratories, Inc., 3 Science Road, Glen-
wood Science Park, Glenwood, Illinois 60425.

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CHILDREN'S MEDICAL CENTER
ANTIMICROBIAL SUSCEPTIBILITY REPORT

CULTURE NO.: _____ NAME _____
DATE OF CULTURE: _____ RECORD NO.: _____
SOURCE: _____ LOCATION: _____
ORGANISM: _____

DRUG	PLATE DILUTION SUSCEPTIBILITY						DISC
Erythromycin (mcg/ml)	N.I.	20	10	5	1.25	<0.3	S I R
Methicillin (mcg/ml)	N.I.	20	10	5	1.25	<0.3	S I R
Penicillin (Units/ml)	N.I.	2.5	1.25	0.6	0.2	<0.08	S I R
Cephalothin (mcg/ml)	N.I.	20	10	5	1.25	<0.3	S I R
Ampicillin (mcg/ml)	N.I.	20	10	5	1.25	<0.3	S I R
Chloramphenicol (mcg/ml)	N.I.	20	10	5	1.25	<0.3	S I R
Kanamycin (mcg/ml)	N.I.	20	10	5	1.25	<0.3	S I R
Polymyxin B (mcg/ml)	N.I.	20	10	5	1.25	<0.3	S I R
Tetracycline (mcg/ml)	N.I.	20	10	5	1.25	<0.3	S I R
Gentamicin (mcg/ml)	N.I.	10	5	2.5	1.25	<0.3	S I R

Resistant Range Susceptible Range

FIG. 1. Facsimile of form used to report antimicrobial susceptibility results. "N.I." stands for no inhibition at the highest concentration of drug tested. MIC's are indicated by circling the appropriate value. Susceptibility with disc agar diffusion testing is reported as either susceptible (S), intermediate (I), or resistant (R).

sterile phosphate-buffered saline solution (pH 7.4). Seed cultures of *Pseudomonas* species are not diluted.

Plates are inoculated with an inoculating apparatus,⁸ thereby allowing the simultaneous inoculation of 32 organisms onto each plate. Thus, a total of 64 organisms can be tested each day, since Gram-negative and Gram-positive sets are inoculated separately. Inoculating wells are filled with the diluted seed cultures with sterile Pasteur pipettes. Each inoculating prong delivers approximately 0.01 ml., containing 10^4 to 10^5 viable organisms. After inoculation, plates are left in the upright position for 30 min. to insure complete absorption of the inocula and then inverted and incubated at 37 C. for 16 hr. The lowest concentration of a given drug resulting in complete inhibition of growth, a barely visible haze of apparent growth, or a single pinpoint colony is reported as the minimal inhibitory concentration (MIC).¹

A facsimile of the antibiotic susceptibility report form is shown in Figure 1. Or-

ganisms with MIC's of ≤ 10 μ g. per ml. with cephalothin, kanamycin, ampicillin, methicillin, and chloramphenicol are defined as susceptible. For gentamicin, polymyxin B, tetracycline and erythromycin, the level of susceptibility is set one dilution lower, at ≤ 5 μ g. per ml. A MIC of ≤ 0.6 units per ml. indicates susceptibility to penicillin.

For quality control, two stock strains of coagulase-positive staphylococci, one strain of *E. coli*, and one strain of *Klebsiella pneumoniae* with known, diverse susceptibility patterns are tested each day. In addition, viable colony counts are performed periodically on diluted seed cultures selected at random to insure that inocula contain the desired number of organisms.

The disk diffusion technic of Bauer and co-workers² is used in place of the agar dilution method in the following circumstances: (1) routinely to test susceptibility of enteropathogenic *E. coli* to neomycin, (2) routinely to test susceptibilities of *Proteus* and *Pseudomonas* species to carbeni-

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cillin, (3) occasionally to test susceptibilities of an isolate available for testing on Saturdays, and (4) occasionally to test susceptibilities of an isolate to infrequently used drugs not included in the test battery.

Evaluation Studies

For the purposes of this report three studies were performed:

(1) Sixty bacteria chosen at random were tested by the broth dilution technic and the results correlated with those previously obtained by routine agar dilution testing. Thirty-five Gram-negative organisms comprising five strains of each of the following seven types of bacteria were tested: *Klebsiella*, *Enterobacter*, *E. coli*, *Ps. aeruginosa*, *Shigella*, *Salmonella* and *Proteus*. Twenty-five isolates of coagulase-positive staphylococci were tested. Broth dilution testing was done in the pediatric infectious disease research laboratories. Antibiotics were diluted serially in screw-top tubes containing 5 ml. amounts of Oxoid Sensitivity Test Broth to obtain the same concentrations present in agar. Initial seed cultures were prepared as they were for the agar dilution method. Diluted seed cultures were made by inoculating 0.01 ml. of the initial seed cultures into 10-ml. portions of sterile phosphate-buffered saline solution (pH 7.4); 0.1 ml. amounts of these cultures were then transferred to the antibiotic-containing broths by sterile pipettes. Viable colony counts were determined for all diluted seed cultures tested by both agar and broth technics to insure that inoculum sizes were comparable. Tubes were incubated at 37 C. for 16 hr. The lowest concentration of antibiotic resulting in an absence of visible growth was recorded as the MIC.

(2) To document that results obtained with commercial antibiotic preparations compare favorably with those obtained using antibiotic laboratory standards, 120 organisms were tested with plates prepared with both forms of drug. Polymyxin B was

not evaluated because a laboratory standard was not available, nor was chloramphenicol tested in duplicate since the laboratory standard is used for routine testing.

(3) A "time-work" study was carried out to determine the amount of time the agar dilution method demands of the laboratory staff during an average week.

Dissemination of Susceptibility Information to Physicians

Copies of the antibiotic susceptibility report forms are sent to the infectious disease consultant once a month. Results are tabulated according to sources of cultures and organisms isolated. Every 6 months (January and August), cumulated summaries of selected information with interpretative comments are sent to the attending physicians and house staff.

Results

Comparison of Broth and Agar Dilution Methods

Results obtained testing Gram-negative organisms by tube and plate methods are compared in Figure 2 (tetracycline, ampicillin, chloramphenicol, and cephalothin) and Figure 3 (polymyxin B, gentamicin, and kanamycin). Except for one isolate tested with chloramphenicol, all organisms had identical MIC's or varied by only one dilution when tested by the two methods.

If it is assumed that the broth dilution technic is the more accurate of the two methods, the results can be evaluated in a more clinically relevant manner by asking the following question: how many organisms tested by the agar technic would have been interpreted by the physician as resistant when the broth method indicated susceptibility, and *vice versa*? For ampicillin and gentamicin, such a discrepancy would not have occurred; for tetracycline, polymyxin B and kanamycin it would have occurred with one isolate each; for cephalo-

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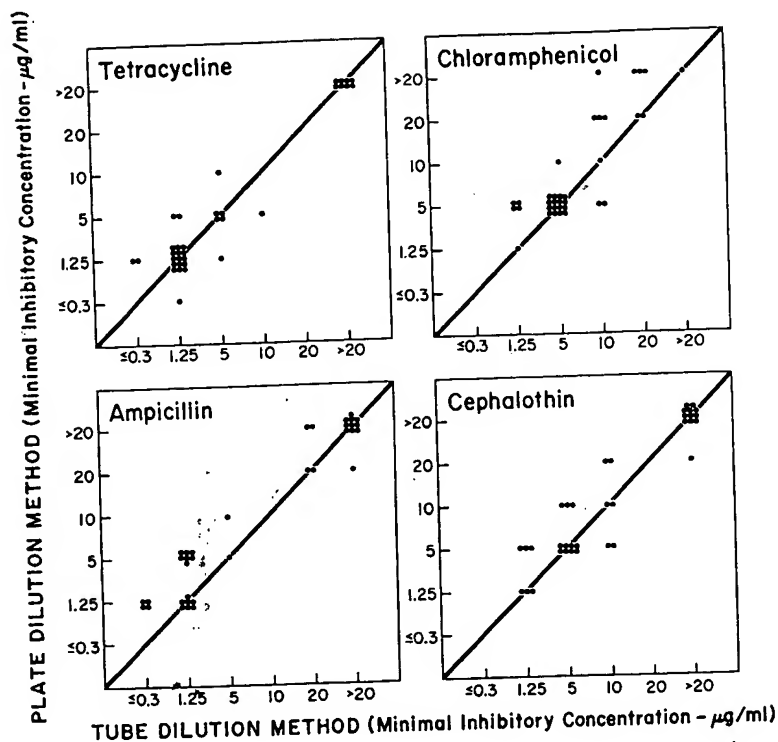


FIG. 2. Correlation of results of agar plate dilution and broth dilution tube methods obtained testing 35 Gram-negative organisms with four antibiotics.

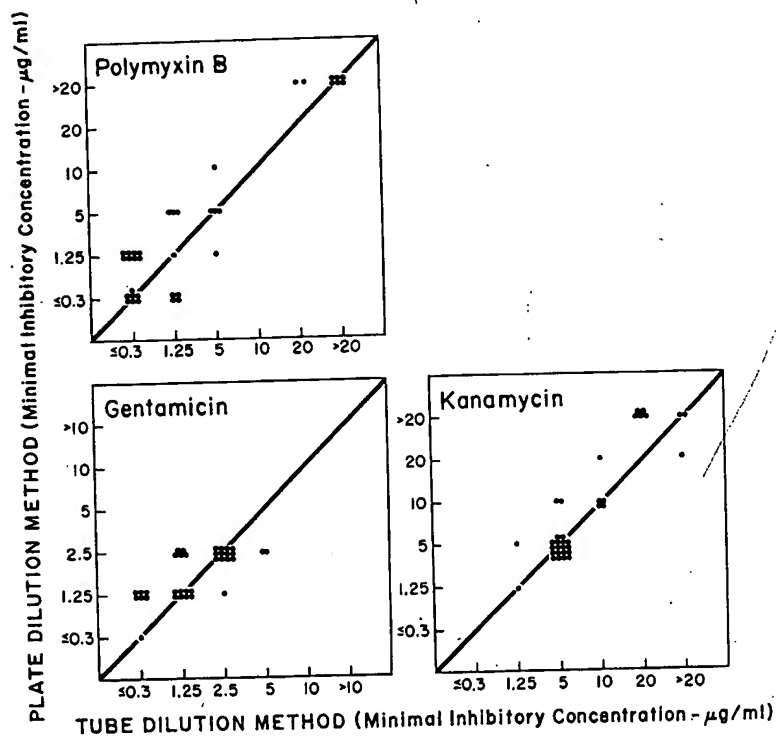
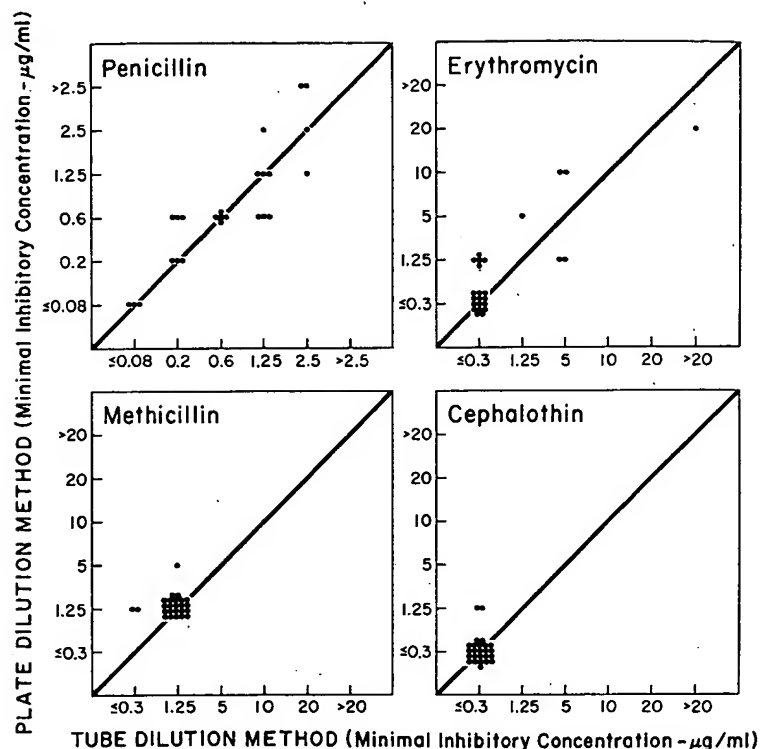


FIG. 3. Correlation of results of agar plate dilution and broth dilution tube methods obtained testing 35 Gram-negative organisms with three antibiotics.

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FIG. 2. Correlation of results of agar plate dilution and broth dilution tube methods obtained testing 35 Gram-negative organisms with four antibiotics.

FIG. 4. Correlation of results of agar plate dilution and broth dilution tube methods obtained testing 25 coagulase-positive staphylococci with four antibiotics.



thin with two isolates; for chloramphenicol with four organisms. In other words, of 135 tests (35 organisms tested with seven drugs) 6.6% (nine tests) were interpreted as resistant by the agar method when they would have been considered susceptible by the broth technic. In only one case (0.7%), with tetracycline, was an organism assumed to be susceptible by agar testing when it was resistant by the broth method.

Results obtained testing coagulase-positive staphylococci by the two methods with penicillin, methicillin, erythromycin, and cephalothin are compared in Figure 4. All strains had identical MIC's or varied by only one dilution when tested by the two methods. Correlation with methicillin and cephalothin was exceedingly accurate, with 88% (22/25) of methicillin tests and 92% (23/25) of cephalothin tests having identical MIC's by the two methods. Of 100 tests (25 organisms tested with four drugs), only 2%, representing two erythromycin

results, would have been interpreted by the clinician as resistant when they were susceptible by the broth method. Conversely, only 3% of tests (all with penicillin) would have been interpreted as susceptible by agar testing when they were resistant by the broth method. Results of gentamicin testing (not depicted graphically) showed that 80% (20/25) of isolates had identical MIC's by the two methods and 20% (5/25) varied by one dilution. In no case would the interpretation of resistance or susceptibility have been at variance by either method.

Comparison of Antibiotic Laboratory Standards and Commercial Antibiotics

Table 1 shows the correlation of the MIC's obtained using both forms of drug. Without exception, all MIC's were identical or varied by only one dilution. Identical results were seen in 90% or more of tests

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Table 1. Correlation of Minimal Inhibitory Concentrations Obtained with Antibiotic Laboratory Standards and with Commercial Antibiotic Preparations for Intravenous Administration

Antibiotic	Number and Type of Organisms Tested	Minimal Inhibitory Concentrations	
		Identical (%)	One-dilution Variation (%)
Gentamicin	70 gram-neg. rods	58	42
Cephalothin	39 gram-pos. cocci		
	70 gram-neg. rods	94	6
Ampicillin	39 gram-pos. cocci		
	70 gram-neg. rods	71	29
Kanamycin	70 gram-neg. rods	90	10
Tetracycline	70 gram-neg. rods	91	9
Erythromycin	50 gram-pos. cocci	78	22
Methicillin	50 gram-pos. cocci	54	46
Penicillin	50 gram-pos. cocci	56	44

with cephalothin, kanamycin and tetracycline; in 71% and 78%, respectively, with ampicillin and erythromycin; and in between 50 and 60% of tests with gentamicin, methicillin, and penicillin. With two antibiotics, gentamicin and ampicillin, the one-dilution variation was almost exclusively in the same direction, with the laboratory standards producing MIC's one dilution lower than the commercial preparations. For gentamicin the figures were 45 of the 46 variations, and for ampicillin they were 18 of 20 variations.

Time-Work Evaluation

The time required by the laboratory staff to perform the procedures connected with the agar dilution method for a typical week is shown in Table 2. The features unique to the method, dilution of antibiotics and use of the replicator, occupy only one-fifth of the total time expended. The other items of work, with appropriate modifications, are necessary for the performance of any testing method.

Six-month Summaries

Summaries of results for the 6-month period from July 1 to December 31, 1971, that

were circulated to the house staff and attending physicians are presented in Tables 3 and 4.

Discussion

The agar dilution method using antibiotic preparations designed for intravenous administration is a reliable, efficient, and financially feasible way to perform routine antibiotic susceptibility testing in our hospital laboratory. Results compare favorably with those obtained with broth dilution testing and with agar dilution testing using antibiotic laboratory standards. Intravenous antibiotic preparations obtained from the hospital pharmacy are recommended for routine agar dilution testing,¹ but variations in potency may occur from one manufacturer to another because of such factors as "overfilling" of vials. Even though it is unlikely that such variations would affect results significantly, it would be ideal if standard laboratory preparations were commercially available from suppliers such as those who manufacture disks. At the present time laboratory standards must be obtained directly from each pharmaceutical house. This would be a major step toward developing the agar dilution method into

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ained with Antibiotic
Preparations

Inhibitory Concentrations

Identical (%)	One- dilution Variation (%)
58	42
94	6
71	29
90	10
91	9
78	22
54	46
56	44

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Discussion

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a fully standardized technic suitable for
widespread use.

The agar dilution method has been ac-
cepted enthusiastically by our laboratory
personnel, who believe, on the basis of past
experience, that agar dilution testing is no
more, and probably less, time-consuming
to perform than the disk agar diffusion
technic of Bauer and associates.³ Although
we did not evaluate the differences between
the agar dilution method and this disk
technic, we feel that it is appropriate to
contrast some of the features of each, since
it is used so extensively. In our opinion,
the agar dilution method possesses several
advantages. To begin with, our dilution
method utilizes Oxoid Sensitivity Test Me-
dium instead of Mueller-Hinton medium.

The Oxoid preparation is a clearer me-
dium that generally yields better bacterial
growth, supports optimal growth of strepto-
cocci without additives, and decreases some-
what the swarming character of *Proteus*
species.⁴ Results of disk testing with the
Oxoid medium have been reported to be
comparable to those obtained with Mueller-
Hinton medium⁴ but it is not recom-
mended for routine use because the disk
technic has been standardized with the lat-
ter medium.

The depth of the agar for the disk tech-
nic is important, and should be 5 to 6 mm.²
Variations in depth may give rise to mis-
leading results, since antibiotics diffuse
downward as well as outward. Depth of the
agar is not a critical factor with the agar
dilution method because the antibiotics are
evenly concentrated throughout the me-
dium. According to Bauer and colleagues,²
poured plates for the disk technic should
be used within 4 days after preparation,
while plates containing antibiotics incor-
porated in agar may be stored longer. The
antibiotics used in our test battery, with
the exception of gentamicin, have been
evaluated by others with respect to their
stability in agar after refrigerated storage

Table 2. Amount of Time Required per Week
to Perform Agar Plate Dilution
Testing*

Procedure	Time (Hr.)
Media and antibiotics	
Preparation of media	1
Dilution of antibiotics	1½
Pouring and labeling of plates	2
Preparation of seed cultures	3½
Performance of tests	
Loading of inoculating wells and replication	1½
Reading of results	1½
Recording and reporting of results	3½
TOTAL	14

* Based on an average of 100 organisms per week.

for various periods of time.⁷ Significant loss
of activity occurred with none after 1 week
and only with penicillin and ampicillin
after 4 weeks of storage. Our experience
with gentamicin shows that it is stable in
agar for at least 10 days.

The inoculum delivered to the agar sur-
face in the disk technic cannot be quanti-
tated as accurately as it can in the agar
dilution method because the amounts of
seed culture adhering to the applicator
swab vary from one culture to another.

In our opinion the procedures connected
with the disks themselves are a vexing part
of the disk technic. They must be stored
properly to avoid loss of potency, expira-
tion dates must be checked, and they must
be placed carefully in appropriate positions
on the agar surface. Furthermore, we be-
lieve that with agar dilution testing reading
results is simpler and more accurate than
with the disk technic. With agar dilution
testing results are read as either "growth"
or "no growth" for a given concentration
of antibiotic. Occasionally a barely visible
haze or a single pinpoint colony may ap-
pear at the site of inoculation; such occur-
rences are interpreted as no growth.⁶ With
the disk technic, precise measurements of
zone diameters must be made, which at

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Table 3. Plate Dilution Antibiotic Susceptibilities of Gram-negative Bacteria Isolated at Children's Medical Center from July 1 to December 31, 1971

Type of Bacteria	Number Tested	Percentage Susceptible to Concentrations of Antibiotic Indicated						
		Cephalothin ≤ 10 µg./ml.	Gentamicin ≤ 5 µg./ml.	Kanamycin ≤ 10 µg./ml.	Polymyxin B ≤ 5 µg./ml.	Ampicillin ≤ 10 µg./ml.	Tetracycline ≤ 5 µg./ml.	Chloramphenicol ≤ 10 µg./ml.
<i>Escherichia coli</i> Enteropathogenic Urine Other sources	78	81	95	87	92	65	74	92
	167	83	99	92	98	75	69	94
	67	87	96	82	96	81	72	94
<i>Proteus</i> Indol-positive Indol-negative	15	20	93	80	0	33	27	60
	64	67	98	92	0	84	5	88
<i>Klebsiella</i>	131	81	98	86	86	10	83	94
<i>Enterobacter</i>	56	16	98	89	63	27	64	83
<i>Shigella</i>	123	95	100	84	99	97	89	97
<i>Salmonella</i>	57	95	98	93	100	86	82	100
<i>Pseudomonas aeruginosa</i>	191	5	99	8	90	8	32	9

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Shigella	123	95	100	84	99	97	89	97
Salmonella	57	95	98	93	100	86	82	100
<i>Pseudomonas aeruginosa</i>	191	5	99	8	90	8	32	9

times may be difficult because of hazy zone perimeters or asymmetrical zone areas even with use of an electronic zone reader. Measurements must then be checked against standards for each antibiotic to determine whether the organism is susceptible, of intermediate susceptibility, or resistant.

Last, we believe that the most important advantage of the agar dilution method is that results are reported as MIC's of a given antibiotic. Thus, clinicians are able to correlate these values with serum levels of antibiotic achievable in patients following various doses given by the various routes of administration.

To avoid problems of acceptance and understanding by physicians, a written explanation outlining the agar dilution method was sent to both the house staff and the attending staff before institution of the technic. No complaints concerning either the type and number of antibiotics tested or the manner in which results are reported have been received. The house staff is carefully instructed regarding the interpretation of minimal inhibitory concentrations in the light of attainable levels of antibiotic in serum and other fluids and the individual patient's disease process. If a member of the attending staff is uncertain how to interpret a result, he usually will consult a house officer or check with a member of the infectious disease service.

The types of antibiotics that are prescribed in our hospital have been influenced by the agar dilution method. We have found that physicians are unlikely to use drugs that are not included in the agar dilution test battery, with the exception of such drugs as neomycin for diarrheal disease due to enteropathogenic *E. coli* and carbenicillin for infections caused by *Ps. aeruginosa* and certain *Proteus* species; disk testing is performed routinely under these circumstances. Physicians may request that disk testing be performed with other agents, but rarely do so.

Table 4. Plate Dilution Antibiotic Susceptibilities of Coagulase-positive Staphylococci and Beta-hemolytic Streptococci Isolated at Children's Medical Center from July 1 to December 31, 1971

Type of Bacteria	Number Tested	Percentage Susceptible to Concentrations of Antibiotic Indicated							
		Penicillin ≤0.6 U./ml.	Methicillin ≤10 μg./ml.	Erythro- mycin ≤5 μg./ml.	Cephalo- thin ≤10 μg./ml.	Genta- micin ≤5 μg./ml.	Kana- mycin ≤10 μg./ml.	Polymyxin B ≤5 μg./ml.	Ampicillin ≤10 μg./ml.
Staphylococci (coagulase-positive) Respiratory tract Skin/eye/ear Tissue/blood	151	73	99	97	99	96	Not tested	Not tested	Not tested
	211	78	99.5	95	99	99	Not tested	Not tested	Not tested
	135	83	100	99	100	99	33	0	0
Streptococci (beta-hemolytic) Not Group A or D Group D	10	100	100	100	100	10	33	0	100
	44	33	30	94	34	23	7	5	36
									93

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It could be argued that other drugs should be added to the test battery and that some currently included should be deleted. We believe that the drugs tested should be appropriate for the particular population of a given hospital. Furthermore, the total number of drugs tested and the antibiotic concentrations evaluated should be realistic so far as the laboratory is concerned. At this point, the drugs included in our battery and the concentrations used are appropriate for our hospital. However, the composition of the battery should be reviewed periodically and changes made when indicated.

The total number of organisms that can be tested each day, including stock control strains, without increasing the amount or cost of materials is 64; 32 in the Gram-negative set and 32 in the Gram-positive set. This potential is sufficient to take care of our needs for the foreseeable future. When the stage is reached where 32 Gram-negative organisms are tested each day, "restraining rings"† will have to be applied to the agar surrounding *Proteus* inocula to inhibit swarming completely. At the present time *Proteus* isolates are segregated to one portion of the plate and spaced appropriately.

Adoption of the agar dilution method for use in hospitals with larger bacteriology services than ours is feasible.⁶ For example, the laboratory of Parkland Memorial Hospital in Dallas has used an agar dilution

method for several years and performs an average of 60 susceptibility tests daily. Until recently plates in that laboratory were inoculated using a swab technique; inoculation is now performed with the replicating device of Steers and associates.⁸

Acknowledgments. Patricia A. Brooks, M.T. (ASCP), Karen B. Moyer, B.S., and Arthur G. Weinberg, M.D., Director of Laboratories, cooperated in this study. Helen T. Kusmiesz, R.N., tabulated the susceptibility information that was sent to the physicians.

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† Available as "Raschig rings" from Scientific Apparatus Co., Bloomfield, N. J.

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